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Single nucleotide polymorphism analysis by allele-specific primer extension with real-time bioluminescence detection in a microfluidic device

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Abstract

A microfluidic approach for rapid bioluminescent real-time detection of single nucleotide polymorphism (SNP) is presented. The method is based on single-step primer extension using pyrosequencing chemistry to monitor nucleotide incorporations in real-time. The method takes advantage of the fact that the reaction kinetics differ between matched and mismatched primer–template configurations. We show here that monitoring the initial reaction in real time accurately scores SNPs by comparing the initial reaction kinetics between matched and mismatched configurations. Thus, no additional treatment is required to improve the sequence specificity of the extension, which has been the case for many allele-specific extension assays. The microfluidic approach was evaluated using four SNPs. Three of the SNPs included primer–template configurations that have been previously reported to be difficult to resolve by allele-specific primer extension. All SNPs investigated were successfully scored. Using the microfluidic device, the volume for the bioluminescent assay was reduced dramatically, thus offering a cost-effective and fast SNP analysis method.

Keywords: Microfluidics; Pyrosequencing; Nucleotide polymorphism; Primer extension; Nucleotides

1. Introduction

Now that the human genome has in principle been sequenced, DNA sequence analysis can be expected to have its greatest impact on the understanding of how genetic variation leads to disease. Interest in the study of naturally occurring sequence variations has increased tremendously in the last few years. The most common type of genetic diversity is the single nucleotide polymorphism (SNP). SNPs are single base pair positions in genomic DNA at which different sequence alternatives (alleles) exist in normal individuals in one or more populations.

A wide variety of techniques have been developed to type SNPs in a high-throughput fashion, including electrophoresis [1,2], the amplification refractory mutation systems (ARMS) [3], Invader [4,5], 5′-nuclease TaqMan [6,7], dynamic allele-specific hybridization (DASH) [8], molecular beacon probes [9], mass spectrometry [10,11], pyrosequencing [12–15], DNA chips [2,16], electric-field-controlled nucleic acid hybridization [17,18], electro-catalysis [19] and bead technology [20,21].

Allele-specific primer extension is a technique that has been employed previously to identify single base variations [3,22,23]. The method exploits the differ-

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ence in rate of extension by a DNA polymerase of annealed primers matched (rapid extension), or mismatched (slow extension) at the 3′ end with the corresponding variable base (SNP) on the sample DNA strand. The allelic variant of an SNP can thus be determined by placing the 3′ end of the primer over the variable position. However, this method suffers from poor discrimination by the DNA polymerase of certain mismatches, such as G:T and C:A [24]. Different solutions have been proposed to improve the discrimination properties of DNA polymerases but all of these procedures include extensive optimization steps. However, it was shown that the reaction kinetics are slower for mismatched configurations as compared with matched configurations and this has been exploited in the apyrase-mediated allele-specific extension (AMASE) assay for successful SNP typing using a real-time bioluminometric approach [25] and on oligonucleotide microarrays [26].

Recently, we presented a preliminary study describing a microfluidic approach for allele-specific extension using fluorescently labelled nucleotides that was used to take advantage of the differences in reaction kinetics [27]. In the work presented here we have further developed our microfluidic approach to allele-specific extension to take advantage of differences between match and mismatch in the initial kinetics of pyrosequencing. Pyrosequencing chemistry involves an enzymatic cascade reaction to monitor nucleotide incorporation in real-time [13]. In this study, four SNPs were investigated with eight allele-specific primers. Three of the SNPs included primer–template configurations giving mismatches that have been previously reported to be difficult to resolve.

2. Materials and methods

2.1. Microfabrication

Standard semiconductor photolithographic techniques and bulk micro-machining of silicon were used to manufacture the device [28]. The front side of the silicon substrate was patterned and etched in a standard inductively-coupled plasma (ICP) etcher (Surface Technology System, Newport, UK) forming the inlet and outlet channels and the reaction-chamber. A Pyrex wafer was anodically bonded to seal the device. The backside was then patterned and etched to create the fluid connectors. Finally, the silicon-glass stack was diced into 9×5-mm chips. External polyethylene tube was attached to the fluid connectors by a melt-on method [28]. Fig. 1 shows a scanning electron microscope (SEM) image of the microfluidic device for trapping beads, including inlet channel, reaction chamber with filter pillars, waste chamber and the outlet channel. The reaction chamber volume used in this study was 50 nl.

2.2. Sample preparation

Three biotinylated polymerase chain reaction (PCR) products containing the SNPs G2215A and T3409C in the ACE gene, and the SNP A1166C in the AT1R gene, respectively, were prepared according to Ref. [29]. One biotinylated PCR product containing the SNP T573C in the AT1R gene was amplified according to Ref. [29] using the primers 5′-GTTTGTGCTTTCCATTATGAGTC-3′ and 5′-b-CATTTCTTGGTTTGTTCTTCTGA-3′.

The biotinylated PCR products were immobilized on non-magnetic streptavidin-coated 5.5-μm beads ($2{\cdot}10^6$ beads/PCR) (Bangs Labs., IN, USA) by incubating 50 μl of PCR product with 20 μl of beads in 50 μl binding buffer (10 m*M* Tris–HCl, 2 *M* NaCl, 1 m*M* EDTA, 0.1% Tween 20) for 30 min

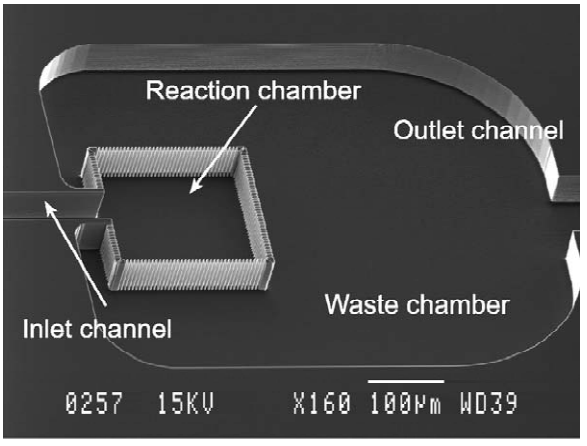

Fig. 1. A scanning electron microscope photograph of the flow-through device.

with mixing. Single-stranded DNA was obtained by alkali elution of the non-biotinylated strand, using 0.2 *M* NaOH, followed by washing in 10 m*M* Tris–acetate, pH 7.6. The beads bearing the immobilized strand were resuspended in 25 μl water and 3 μl annealing buffer (10×, 100 m*M* Tris–acetate, pH 7.6, 50 m*M* magnesium acetate) was added. The suspension was divided into two wells of a microtiter plate, and 2 μl (3 μ*M*) of allele-specific primers differing in their 3′-ends were added to each well. The allele-specific primers were as shown in Table 1. Hybridization was performed by incubation at 95 °C for 4 min and then cooling to room temperature.

2.3. Analysis of allele-specific extension

Beads carrying ~0.03 pmol immobilized single-stranded DNA with annealed primer (the substrate) were trapped in the reaction chamber (50 nl) by applying negative pressure (suction from the outlet; pump from Parameter, Stockholm, Sweden). The pyrosequencing mixture (200-nl volume) was dispensed using an automatic inkjet dispenser. The stock pyrosequencing mixture contained exonuclease-deficient (exo-) Klenow DNA polymerase (Amersham Biosciences, Uppsala, Sweden), 0.7 U/μl; purified luciferase (BioTherma, Dalarö, Sweden), 27 ng/μl; recombinant ATP sulfurylase, 1.7 mU/μl; 75 m*M* Tris–acetate (pH 7.6); 0.5 m*M* EDTA; 35 m*M* magnesium acetate; 0.1% (w/v) bovine serum albumin (BioTherma); 0.7 m*M* dithiothreitol; 10 μ*M* adenosine 5′-phosphosulfate (APS); polyvinylpyrrolidone, 0.4 μg/μl (360 000); D-luciferin (BioTherma), 100 ng/μl; dATP alpha S, 100 μ*M*; dCTP, dGTP and dTTP, 30 μ*M*. As the reaction mixture flowed into the reaction chamber and came in contact with the DNA immobilized on beads, incorporation took place and the emitted light was detected by a charge-coupled device (CCD) camera. The extension procedure was carried out in a dark box and the data were obtained in Excel and graphic formats. The reaction was terminated after 10 s by applying backpressure. After each reaction, the microfluidic device was regenerated by applying pressure at the outlet to remove the beads from the reaction chamber.

3. Results

The principle of primer extension linked to pyrosequencing is shown in Fig. 2. Two primers, each designed to match one allele perfectly but mismatch the other allele at the 3′-end, were used. If the 3′-end of the primer matches the DNA template (Fig. 2a) the DNA polymerase will catalyze the incorporation of the nucleotides into the DNA strand. Thus, the light signal indicates that the DNA polymerase has used the nucleotides to extend a matched primer. If the 3′-end of the primer does not match the DNA template (Fig. 2b), then the rate of extension of the primer by the DNA polymerase will be significantly lower and very little light will be produced.

The principle described above was used to analyze four different SNPs using four pairs of sequencing primers (Table 1). Thus the following mismatches were investigated: A:C (or C:A), G:T (or T:G), A:G and C:T. Fig. 3 shows the results of allele-specific

Table 1
The SNPs analysed and extension primers used in the study

Gene	Position	SNP (U)	SNP (L)	Extension primer 1 (5′→3′)	Mismatch	Extension primer 2 (5′→3′)	Mismatch
ACE	G2215A	A/G	T/C	AGAACGGGCAGC**A**	A:C	AGAACGGGCAGC**G**	G:T
ACE	T3409C	T/C	A/G	TCTGCTCCAGGTACTT**C**	C:A	TCTGCTCCAGGTACTT**T**	T:G
AT1R	T573C	T/C	A/G	CCCAAAATTCAACCCT**T**	T:G	CCCAAAATTCAACCCT**C**	C:A
AT1R	A1166C	A/C	T/G	CTTCACTACCAAATGAGC**A**	A:G	CTTCACTACCAAATGAGC**C**	C:T

SNP(U), the variation as reported in the literature (upper strand); SNP(L), the variation in the immobilised lower strand that was analysed using one of the extension primers. Extension primers 1 and 2 are shown with the 3′ base in bold, underlined, that created a mismatch as indicated with one allele of the SNP. The primers gave either a match or a mismatch or both (in the case of a heterozygote) depending on the genotype of the sample analysed and the primer used.

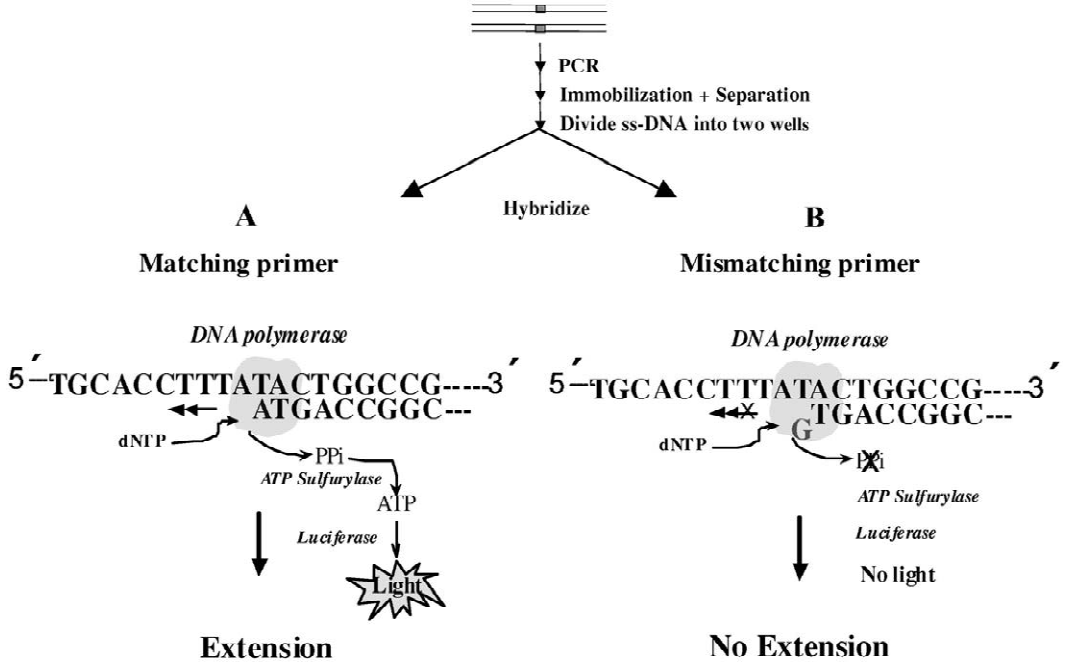

Fig. 2. The principle of allele-specific extension by pyrosequencing chemistry. PCR is used to amplify a region containing a polymorphic position and single-stranded DNA is obtained after immobilization on beads. The target single-stranded DNA is divided into two tubes. Each tube receives one of two allele-specific primers (that match at their 3′-ends with one of the two alleles) and these are hybridized to the target DNA. A pyrosequencing mixture (excluding apyrase) with the four nucleotides is added to the primer–template. (a) A 3′-end matched primer–template results in extension of the primer by DNA polymerase and release of pyrophosphate (PPi) upon incorporation of nucleotides. The PPi is converted to ATP and then to light by sulfurylase and luciferase, respectively. Thus the light signal indicates that the DNA polymerase has used the nucleotides to extend a matched primer–template. (b) If the 3′-end of the primer does not match the DNA template then the rate of extension of the primer by the DNA polymerase will be significantly lower and very little light will be produced.

extensions for all three variants of the SNP AT1R (T573C). The SNP position involves either an A or G base (Table 1). The raw signal is plotted in Fig. 3a–c and the corresponding derivative is plotted in Fig. 3d–f. In the case of homozygous A and G (Fig. 3a,c), the matched variant resulted in a rapidly increasing light signal that was clearly distinguishable from the slow rate of the mismatched variant. The mismatched signals (Fig. 3a,c) were almost constant at a low level, revealing that the DNA polymerase had difficulty in extending the mismatched primer–template during the initial stage of the reaction. In the case of the heterozygous sample (Fig. 3b), the two primers each matched 50% of the template molecules (one of the two alleles) and therefore the two extension signals had almost the same pattern (light intensity increased with time at almost the same rate). The corresponding derivative (slope of the rate of light production), indicating the reaction rate of the extension (Fig. 3d–f), shows very clearly the patterns described above for the raw data.

The discriminatory power of the assay for the two alleles can be measured by taking the ratio between extension signals for matched and mismatched primers. The extension signal and the corresponding derivative were extracted and the extension ratio was calculated by taking the ratio of the high versus low signal. A summary of the ratios calculated from the total signals and the corresponding derivatives for the four SNPs is presented in Table 2. In our assay, ratios of ≤ 1.5 were interpreted as heterozygous with ratios of ≥ 4 as homozygous. As can be seen in Table 2, all the SNPs investigated were correctly scored.

Tests were performed that confirmed that the best

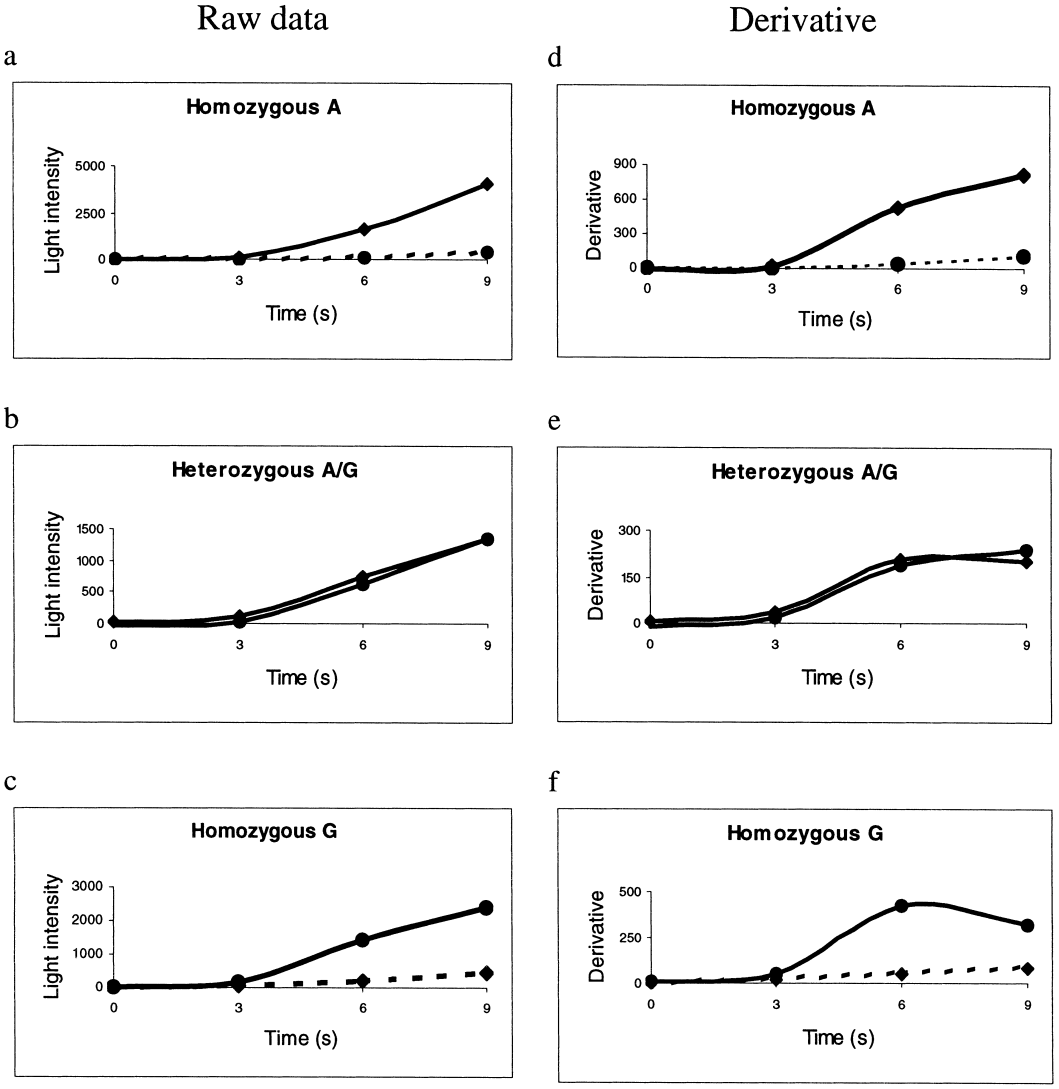

Fig. 3. The results of the bioluminescent allele-specific extensions in the microfluidic device for all three possible genotypes of the SNP at AT1R (T573C). The charts a–c show raw data, and d–e show the corresponding first derivative. In the homozygous samples (a, d and c, f), the signals from matched primers are shown as continuous lines. Signals from mismatched primers are shown as broken lines. In the heterozygous samples (b, e), both samples are shown as continuous lines.

result (resolution) was obtained at the initial stage of the reaction. Fig. 4 shows a real-time extension over a period of 1 min for the homozygous SNP AT1R (T573C). The ratio for the raw signal was 1.3, scoring the homozygous incorrectly as heterozygous. This was in contrast to the correct scoring obtained after 6 s (Fig. 3c), which had a ratio of 6.9. The microfluidic system was therefore designed to terminate the reaction after 10 s by applying pressure at the outlet.

4. Discussion

Comparison of genomic DNA sequences in individuals has revealed many positions at which two

Table 2
Summary of extension results

Gene and sample	SNP variant	Extension primer–template configurations		Extension ratio	
		Primer 1	Primer 2	Raw data	Derivative
ACE (G2215A)		A	G		
(T/C)	T/T	Match	Mismatch	10.5	19.6
	T/C	Match	Match	1.1	1.4
	C/C	Mismatch	Match	16.4	17.2
ACE (T3409C)		T	C		
(A/G)	A/A	Match	Mismatch	5.7	10.2
	A/G	Match	Match	1.2	1.3
	G/G	Mismatch	Match	9.6	9.1
AT1R (T573C)		T	C		
(A/G)	A/A	Match	Mismatch	12.9	13.7
	A/G	Match	Match	1.1	1.2
	G/G	Mismatch	Match	6.9	9.1
AT1R (A1166C)		A	C		
(T/G)	T/T	Match	Mismatch	5.1	14.1
	T/G	Match	Match	1.1	1.2
	G/G	Mismatch	Match	5.4	9.2

or more different sequence alternatives can occur. The typical frequency with which one observes these single nucleotide polymorphisms (SNPs) in the human genome is of the order of 1/1000 bp [30–35]. This number accounts for more than three million SNPs in the human genome [10]. The hope that SNPs will help in the identification of genes that underlie complex diseases has increased the demand for efficient methods to type and assess the biological impact of these kinds of genetic variations.

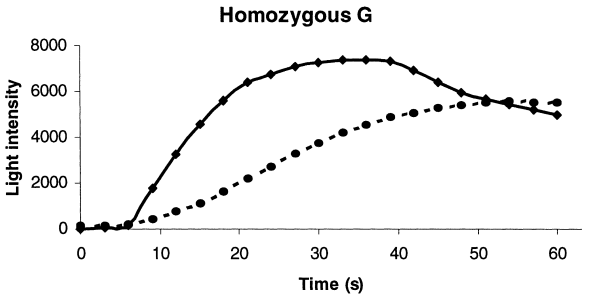

Fig. 4. Bioluminescent allele-specific extension for homozygous (G) of the SNP at AT1R (T573C). The reaction was allowed to run for 1 min. The raw data from the matched primer are shown as a continuous line and the signal from the mismatched primer is shown as a broken line.

New genotyping methods that are high throughput, accurate and cheap are therefore urgently needed.

This report presents a miniaturized microfluidic approach for SNP genotyping by allele-specific primer extension. Allele-specific primer extension using alternating primers at the 3′-end is based on the accuracy of nucleotide incorporation by DNA polymerases and has previously been described to analyze single base variations [3,36], but with varying degrees of success. This is thought to be due to the inability of the DNA polymerase to fully discriminate between certain mismatches, leading to false positive results. However, it was recently shown that the reaction kinetics are slower for mismatched primer–template configurations compared with matched configurations [25]. It is this phenomenon that is further exploited in the current study, but in a miniaturized microfluidic format.

In recent years, microfabricated systems capable of performing biological analysis have been the subject of numerous studies. Using standard photolithographic techniques, network of micrometer-sized channels can be etched into a variety of substrates, most frequently glass or silicon. We have previously evaluated a micromachined microfluidic flow-through device for SNP analysis by employing

pyrosequencing chemistry [37]. As the reaction mixture was loaded manually, the beginning of the real time reaction could not be monitored. As a consequence, it was difficult to score certain mismatches correctly. However, we demonstrated the concept of using the microfluidic approach as a means to increase the sequence specificity of DNA polymerase using fluorescently labelled nucleotides [27], as the detection could be separated from the reaction. To take full advantage of the initial reaction kinetics in the microfluidic approach, a real-time detection method is required. In the current study, emphasis has been placed on the chemistry, fluidics and detection methods to enable real-time monitoring of the initial extension reaction as a method for accurately scoring SNPs.

Four different SNPs have been investigated with eight alternative 3′-end primer–template configurations (Table 1). The discriminatory power of the DNA polymerase was calculated by taking the ratio between matched and mismatched primers extension signals (Table 2). In our assay, ratios of ≤ 1.5 were interpreted as heterozygous with ratios of ≥ 4 as homozygous. The results for all variants are shown in Table 2. As can be seen in Table 2, all the SNPs investigated were correctly scored by this technique. Certain mismatches, such as G:T and C:A, have been reported to be poorly discriminated by certain DNA polymerases [24]. To prove the accuracy of our system, three out of four SNPs included primer–template configurations that have been reported as mismatches that are difficult to resolve (three C:A and three G:T mismatches). As can be seen in Table 2, all six ratios originating from these mismatches resulted in correct genotyping. The derivative illustrates the reaction rate and the difference in reaction kinetics showed that the matched configurations were rapidly extended while the mismatched configurations were discriminated by the DNA polymerase at the initial stage of the reaction. In general, the highest discriminatory power was obtained by using the ratios of the first derivative of the reaction rate.

The power of the method presented here is the use of initial reaction kinetics as a means to differentiate primer–template configurations. As can be seen in Fig. 4, if the reaction of the mismatch is given time, the DNA polymerase starts to extend mismatch configurations. In the worst case, exemplified in Fig. 4, the signal for mismatch reaches almost as high as that for the matched configuration within 1 min, scoring it as false heterozygous (ratio of 1.3). Thus endpoint analysis, even after a relatively short time, would not discriminate between difficult mismatches. This is in agreement with observations reported from other groups measuring endpoint signals. In the apyrase-mediated allele-specific extension (AMASE) by Ahmadian et al. [25], apyrase was introduced to degrade the nucleotides when the reaction kinetics were slow (due to mismatch primer) whilst leaving the rapid kinetics of matched primer extension relatively unaffected. This allowed them to perform endpoint analysis. In our assay, it took ~6 s to obtain the highest ratios based on raw data and the derivatives. Therefore, the microfluidic approach was designed to terminate the reaction after 10 s, simply by applying pressure from the outlet and permitting the fluid to flow through. Thus, we show here that no additional optimization steps, such as introducing artificial mismatched bases [38], or the use of apyrase to increase the extension specificity, are necessary to accurately and rapidly score SNPs by allele-specific primer extension.

The microfluidic device presented here has enabled a dramatic reduction of the volume used. The reaction chamber containing the DNA template volume was 50 nl and the reagent volume dispensed was 200 nl. This can be compared with 10–50 μl used in commercial pyrosequencing instruments manufactured by Pyrosequencing. One of the main advantages with microfabricated microfluidic devices is that they can be mass-produced as arrays at low cost. To enable high throughput SNP analysis, a chip with an array of filter-chambers [39] could be used. Since the detectable product of primer extension (pyrophosphate, PPi) is present in the liquid phase, it is important to control the fluidics in order to develop an array-based luminometric assay. This must be accomplished by precise liquid handling. Fig. 5 shows a picture from a reaction followed in real-time. The light spot is centered on the reaction chamber. Arrays of filter-chambers, easily controlled from the same outlet [39], could therefore be used since there is no diffusion of light to the outlet, minimizing the risk of cross-contamination of light signals between different chambers. In addition, the high signal obtained should allow further miniaturi-

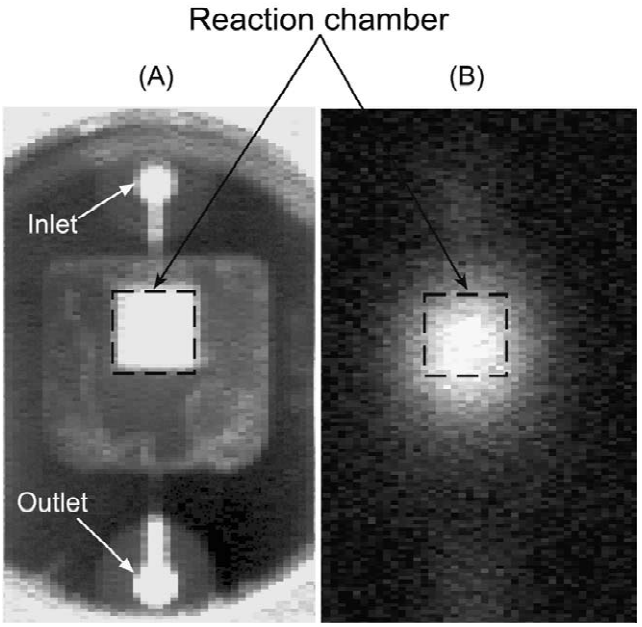

Fig. 5. Image of (A) the filter-chamber with DNA immobilized on beads captured in the reaction chamber before the reaction and (B) light emission during the reaction.

zation of the device and the microfluidic platform developed here offers additional opportunities, such as integration of sample preparation.

In conclusion, a fast microfluidic real-time method for scoring SNPs by allele-specific primer extension has been developed. We show that no additional optimization steps, or the use of apyrase, are necessary to accurately score SNPs by monitoring the initial kinetics in real time. Furthermore, the microfluidic device has reduced the reaction volume dramatically and opens up the possibility of performing SNP analysis in arrays of filter-chambers.

Acknowledgements

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